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**USAMRMC ltr, dtd 15 May 2003**

AD_____

GRANT NUMBER DAMD17-98-1-8122

TITLE: Centrosome Hypertrophy Induced by p53 Mutations Leads to Tumor Aneuploidy

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REPORT DATE: June 1999

TYPE OF REPORT: Annual Summary

PREPARED FOR:
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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# REPORT DOCUMENTATION PAGE

*Form Approved*
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| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | June 1999 | Annual Summary (1 Jun 98 - 31 May 99) |

**4. TITLE AND SUBTITLE**
Centrosome Hypertrophy Induced by p53 Mutations Leads to Tumor Aneuploidy

**5. FUNDING NUMBERS**
DAMD17-98-1-8122

**6. AUTHOR(S)**
Wilma L. Lingle, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Mayo Foundation
Rochester, Minnesota 55905

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

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**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200 words)*

This research is designed to test the hypothesis that aneuploidy in some breast tumors is caused by centrosome abnormalities induced by alteration in p53 function. Specific mutations in p53 associated with breast cancer, aneuploidy, and centrosome abnormalities will be identified. To test the effects of specific p53 mutations on centrosomes, normal mammary epithelial cells will be transfected to over-express mutant p53 and then monitored for changes in ploidy and centrosome structure and function. It is expected that significant novel information will emerge from these studies. First, tumor aneuploidy will be related to a spectrum of p53 mutations, allowing association of specific mutations with specific tumor phenotypes. Second, over-expression of specific p53 mutations on normal human mammary epithelial cells will directly test the hypothesis that some mutations affect centrosome structure and function, resulting in aneuploidy. If specific p53 mutations lead to aneuploidy by affecting centrosomes, then the possibility arises for development of new therapies that target centrosome function. To date, it has been shown that a specific centrosome abnormality, namely excess pericentriolar material, is associated with abnormal mitoses more than other centrosome abnormalities. A p53 mutation that increases the frequency of abnormal mitoses in transfected cells has been identified.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| Breast Cancer, p53, centrosomes, aneuploidy | 46 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | limited |

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William L. Ji 6/27/99

PI - Signature Date

## Table of Contents

## Introduction

The research in this proposal is designed to test the hypothesis that aneuploidy in some breast tumors is caused by centrosome abnormalities which are induced by alteration in p53 function. Specific mutations and expression alterations in p53 that are associated with breast cancer, aneuploidy, and centrosome abnormalities will be identified. In order to test whether or not a specific p53 mutation affects centrosome structure and function, primary cultures of normal mammary epithelial cells will be transfected to over-express the mutant p53. These transfected cells will then be monitored for changes in ploidy and centrosome structure and function. It is expected that significant novel information will emerge from these studies. First, tumor aneuploidy will be related to a spectrum of p53 mutations or expression alterations. Insights into the function of p53 will be gained by associating specific mutations with specific tumor phenotypes. An important aspect is that this portion of the research will be performed on human tumors rather than in animal models or established cell lines. Second, the effects of the mutations will be tested on primary cultures of normal human mammary epithelial cells. This portion of the research will actually test the hypothesis that some p53 mutations directly affect centrosome structure and function, resulting in aneuploidy. If specific p53 mutations lead to aneuploidy by affecting centrosomes, then the possibility arises for development of new therapies that target centrosome function. Cultures of primary mammary epithelial cells are being used instead of established cell lines, even though primary cells have a limited number of passages before they begin to senesce. The increased validity of results from non-immortalized primary cells warrants the technical challenge their use imposes.

## Annual Summary

### Training

I have continued training sessions in the Surgical Pathology Laboratory at one of the Mayo-associated hospitals. On average, these sessions occur once per quarter. In these sessions, I have gained hands-on experience with gross dissection of human tissues and have observed the process of diagnosis using frozen sections. I also have had several training sessions with staff pathologists to diagnose breast tissues using H&E stained preparations of paraffin embedded tissues. These sessions have been very helpful for interpreting the results of my research.

Prior to funding of this proposal, I did attend the Molecular Biology and Pathology of Neoplasia AACR Workshop in summer 1997, as was indicated in the proposal. This intense workshop provided a valuable broad background in cancer pathobiology.

In June of 1998, I attended a 10-day workshop on "3D Microscopy of Living Cells". During this workshop, I gained hands-on experience using state-of-the-art microscopes and software. The Mayo Microscopy Core Facility has since purchased some of this equipment, including a Zeiss 510 LSM confocal microscope (which is capable of UV excitation and has three detectors) and the ancillary equipment needed for live cell observation. This equipment is being used for this research.

Much of my training has been technical. I have gained considerable experience with molecular biology techniques including creating site-directed mutants and transfecting primary cells. This area in particular is one in which I needed more training to become competitive in the field of breast cancer research. Training in this area will continue as this project progresses.

### Research Accomplishments

Due to the anticipated establishment of a DNA Chip Core Facility at the Mayo Clinic in fall of 1999, I have chosen to revise my Statement of Work. This Core Facility will be used for p53 mutation screening as originally included in Task 1. It was felt that efficiency and accuracy of p53 mutation detection available through the DNA Chip Core Facility would warrant alteration in the order in which the Tasks are performed. Also, in response to a suggestion in the review of the proposal, the tumors will not be pre-selected based on ploidy prior to p53 mutation analysis. Tasks have been rearranged in the revised Statement of Work, but no Tasks have been omitted. A revised Statement of Work is presented at the end of this summary. Research Accomplishments to date reflect the Tasks as outlined in the original and revised Statements of Work.

*Task 1 (originally Task 2) - Quantification of structural and functional centrosome alterations (months 1-12).* Approximately 26 tumor and 13 benign tissues have been analyzed for centrosome volume and 34 tumor and 13 benign tissue have been analyzed for MT nucleation capacity. Correlative light and electron microscopy analysis of 6 benign tissues and 28 tumors has revealed that tumors with centrosome abnormalities have higher proliferative, mitotic, and

abnormal mitotic indices than do benign tissues or tumor tissues with normal centrosomes. **Interestingly, one specific centrosome abnormality, excess pericentriolar material, is associated with the highest frequency of abnormal mitoses. These data are included in a manuscript accepted for publication in the American Journal of Pathology (Appendix 3).** This Task is near completion.

*Task 2 (originally part of Task 1) – Screen tissues for aneuploidy (months 10-18).* To date, approximately 35 benign and tumor tissues have been analyzed for ploidy using FISH analysis of chromosomes 3,7,and 17. **All benign tissues were diploid, 2 of 19 tumors were diploid or near diploid, while 17 were aneuploid.** The methods in use have been changed from the original proposal to yield specific information on chromosome 17, which is the location of the p53 gene. This Task is ongoing.

*Task 3(extracted from original Tasks 4 and 5) – Trial site-directed mutagenesis (months 4 and 10) and trial transfection (months 10-14).* A p53 mutated from glycine to serine at amino acid 245 was selected based on its occurrence in Li-Fraumeni families having a high incidence of breast cancers. This mutation was engineered using a Stratagene "Quikchange" mutation kit. Normal human mammary epithelial cells were transfected with mutant p53, wild-type p53, and vector alone. A new transfection agent, GenePorter™ (Gene Therapy Systems, Inc.) has yielded up to 90% efficiency in the trial studies (Figure 1). **Initial results show that cells transfected with the p53 mutant develop a phenotype consistent with the hypothesis, namely centrosome and mitotic spindle abnormalities are present at a much higher frequency in the presence of mutant p53 than they are in normal cells (Figure 1).** Analysis of the transfectants is ongoing.

*Task 4 (originally part of Task 1)– p53 mutation/immunohistochemistry status (months 16-30).* Not yet begun. This was moved from Task 1 in order to take advantage of the Mayo DNA Chip Core Facility that will be operational in late 1999.

*Task 5 (originally Task 3) – Analysis of data from Tasks 1,2, and 4 (months 31-33).* Not yet begun.

*Task 6 (originally Task 4) – Site-directed mutagenesis of p53 using mutant identified in Task 5 (months 33- 36).* Not yet begun.

*Task 7 (originally Task 5) – Transfection and monitoring experiments (months 35-46).* Not yet begun.

*Task 8 (originally Task 6) – Data analysis and manuscript preparation (months 38-48).* Not yet begun.

## Revised Statement of Work

*Task 1- (originally Task 2)*
Quantification of structural and functional centrosome alterations (months 1-12).

*Task 2 – (originally part of Task 1)*
Screen tissues for aneuploidy (months 10-18).

*Task 3 – (extracted from original Tasks 4 and 5)*
Trial site-directed mutagenesis (months 4, 10) and trial transfection (months 10-14).

*Task 4 – (originally part of Task 1)*
p53 mutation/immunohistochemistry status (months 16-30). Mutation screening will be done with DNA chip technology.

*Task 5 – (originally Task 3)*
Analysis of data from Tasks 1,2, and 4 (months 31-33).

*Task 6 – (originally Task 4)*
Site-directed mutagenesis of p53 mutation identified in Task 5 (months 33-36)

*Task 7 – (originally Task 5)*
Transfection and monitoring experiments (months 35-46).

*Task 8 – (originally Task 6)*
Data analysis and manuscript preparation (months 38-48).

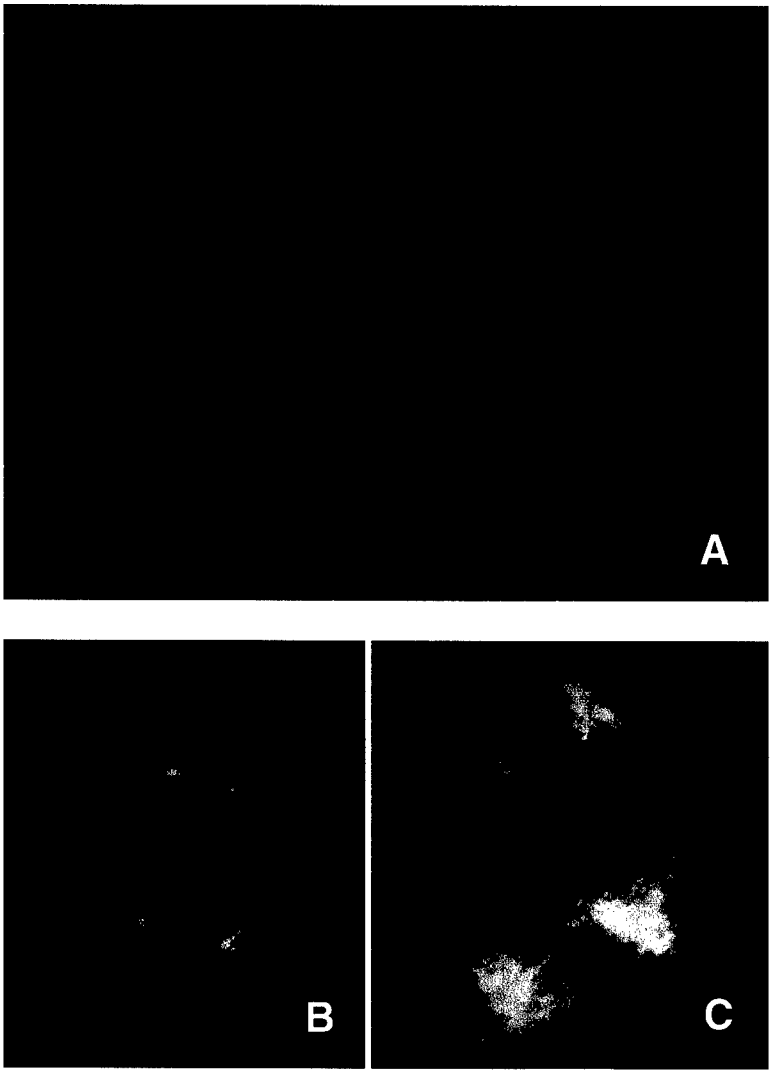
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**Figure 1. A. Transfection Efficiency.** These cells were transiently transfected with wild-type p53 48 hr prior to fixation and immunolabeling. All the cells in this representative field of view were expressing p53 (red). The p53 signal is primarily in the nuclei. The combination of the blue signal from DNA-labeling Hoechst 33342 with the p53 red signal produces the magenta color present in this figure. **B. Vector-alone Transfection Phenotype.** This cell, transfected with vector alone, has a normal bipolar spindle on either side of the condensed chromosomes aligned on the metaphase plate (blue). HsEg5 epitope (green) shows the normal distribution at spindle poles and portions of the microtubules closest to the spindle poles. **C. Mutant p53 Transfection Phenotype.** This cell was transfected with mutant p53. The HsEg5 antibody (green) reveals at least four spindle poles and a mal-formed metaphase plate (blue).

## Appendices

[redacted]

### 1) Key Research Accomplishments

- Excess pericentriolar material is a specific centrosome defect associated with an increased frequency of abnormal mitoses in human breast tumors.
- A specific p53 mutation (glycine to serine at amino acid 245) induces abnormal centrosome structure and function upon transfection of primary normal human mammary epithelial cells.

### 2) Reportable Outcomes

- Manuscript accepted for publication: American Journal of Pathology (pending minor revisions), "Altered Centrosome Structure is Associated with Abnormal Mitoses in Human Breast Tumors" Lingle, WL and Salisbury, JL. See appendix 3.
- Seminar presented: GI Unit Scientific Meeting, Mayo Clinic March 26, 1999 "Aberrant Structure and Function of Centrosomes in Human Breast Tumors".
- Funding applied for: DOD Breast Cancer Research Program 1999 Idea Award, "Regulation of Mitotic Spindle Apparatus Structure and Function by BRCA1-, BRCA2-, and p53-containing protein complexes".
- Promotion received: from Senior Research Fellow to Associate Consultant, effective March 1, 1999.

# Altered Centrosome Structure is Associated with Abnormal Mitoses in Human Breast Tumors

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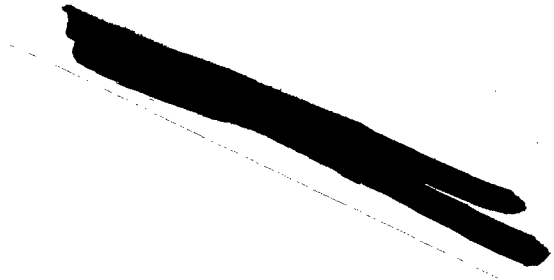

17 text pages

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Running head: Altered Centrosomes in Breast Tumors

Supported by grants from the National Cancer Institute (CA72836 and CA09441), Department of Defense (DAMD17-98-1-8122), and by the Mayo Clinic Foundation

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## Abstract

Centrosomes are the major microtubule organizing center in mammalian cells and establish the spindle poles during mitosis. Centrosome defects have been implicated in disease and tumor progression and have been associated with nullizygosity of the p53 tumor suppressor gene. In the present ultrastructural analysis of 31 human breast tumors, we found that centrosomes of most tumors had significant alterations compared to centrosomes of normal breast tissue. These alterations in included: 1) supernumerary centrioles; 2) excess pericentriolar material; 3) disrupted centriole barrel structure; 4) unincorporated microtubule complexes; 5) centrioles of unusual length; 6) centrioles functioning as ciliary basal bodies; and 7) mispositioned centrosomes. These alterations are associated with changes in cell polarity, changes in cell and tissue differentiation, and chromosome missgegration through multipolar mitoses. Significantly, the presence of excess pericentriolar material was associated with the highest frequency of abnormal mitoses. Centrosome abnormalities may confer a mutator phenotype to tumors, occasionally yielding cells with a selective advantage that emerge and thrive, thus leading the tumor to a more aggressive state.

## Introduction

Checkpoints monitor the nuclear cycle and signal progression following proper completion of earlier stages of the cell cycle.[1] Differentiation, cell proliferation, and programmed cell death are normal outcomes of checkpoint surveillance. In cancer, disregulation of the cell cycle can result in either a decrease in the rate of cell death or an increase in the rate of cell division, and thereby lead to tumor growth. The orderly duplication of the centrosome, once and only once in each cell cycle, and the formation of a bipolar mitotic spindle are key cell cycle checkpoints leading to successful cell division. The importance of the centrosome in the development of malignant tumors was suspected first by Boveri [2] nearly one hundred years ago. More recently, centrosome defects have been implicated in disease and tumor progression.[3-13] Defects in centrosome duplication, alteration in centrosome microtubule nucleation capacity, and inappropriate phosphorylation of centrosome proteins were first described for human breast tumors [14] and subsequently, centrosome anomalies were reported for other tumors.[15-17] Recent evidence suggests that elevated Aurora kinase or STK15 activity may play a key role in acquisition of at least some of these centrosome defects during tumor progression.[18]

The centrosome is the major microtubule organizing center in mammalian cells which regulates the number, stability, polarity, and spatial arrangement of microtubules in interphase cells. [19, 20] Thereby, the centrosome and microtubules play a role in maintaining overall cell polarity, they provide an architectural framework for directed organelle transport, and they participate in cell shape and movement.

The interphase centrosome consists of a pair of orthogonally oriented centrioles surrounded by a pericentriolar matrix. Duplication of the centrosome begins during S phase of the cell cycle when the two centrioles lose their orthogonal arrangement prior to the formation of

a procentriole or "bud" closely associated with the proximal end of each of the original centrioles. The procentrioles lengthen during S and G2, so that by prophase the cell contains two diplosomes, that is two orthogonal pairs of full-length centrioles.[21-24] At the onset of prophase, the diplosomes, along with associated pericentriolar material, move to opposite sides of the nucleus and establish the bipolar mitotic spindle.[25]

We recently have shown that the centrosomes of high-grade breast cancers do not follow this program of events.[14] In breast tumor cells, centrosome duplication is uncoupled from the cell cycle, resulting in cells with numerous centrosomes, many of which are larger than normal. Tumor centrosomes typically show inappropriate levels of phosphorylated proteins, in contrast to normal centrosomes that contain increased levels of phosphorylated proteins during mitosis.

Here we compare the ultrastructure of centrosomes of normal breast epithelial tissues and breast adenocarcinomas. These studies reveal dramatic abnormalities in the centrioles and centrosomes of breast tumor cells. These abnormalities include: 1) supernumerary centrioles, 2) excess pericentriolar material, 3) disrupted centriole barrel structure, 4) unincorporated microtubule complexes, 5) centrioles of unusual length, 6) centrioles functioning as ciliary basal bodies, and 7) mispositioned centrosomes. Structural centrosome abnormalities, most notably excess pericentriolar material, were associated with an increased frequency of abnormal mitoses as assessed by Ki-67 immunolabeled paraffin sections of the same tumors. The relevance of centrosome structure with regard to cell polarity, differentiation, bipolar and multipolar mitosis, and tumor progression is discussed.

## Materials and Methods

### Tissues

Tissues from forty-five consecutive mastectomy and lumpectomy surgeries were collected according to an Institutional Review Board-approved protocol. Tissues were omitted from the analysis if patients had received previous chemotherapy or radiation therapy (N=6), did not include primary invasive tumor (N=4), were poorly preserved (N=3), or were from male patients (N=1). The remaining 31 tumors, which included two grade 2, nine grade 3, and twenty grade 4 specimens (Mayo histologic grading scale), were analyzed. Two normal tissues, from breast reduction surgeries, were also analyzed.

### TEM Processing and Observation

Tissues were cut into small pieces and placed in fixative (4% formaldehyde, 1% glutaraldehyde in sodium phosphate buffer, pH 7.2) at 4° for up to 36 hours. Tissues were further processed by post-fixation in osmium tetroxide, en bloc staining with uranyl acetate, dehydration in ethanol, and embedment in epoxy resin. Thin sections were post-stained with lead citrate and examined using a Philips CM10 Biotwin transmission electron microscope (Philips Electronic Instruments, Mahwah, NJ). Tissues were categorized according to centrosome location, number of centrioles in thin section, qualitative level of pericentriolar material, presence and arrangement of centriolar appendages, presence of primary cilia, variations on centriolar structure, and multipolar mitotic figures.

### Light Microscopy and Mitotic Index Determination

Portions of tissues also were formalin fixed and paraffin embedded for light microscopy.

Sections were immunostained using MIB-1 antibody against Ki-67 (DAKO Corp., Carpinteria, CA). Ki-67 is a nuclear antigen that is present in late G1, S, G2, and mitotic cells, but is lacking in G0 and early G1 cells. Condensed chromosomes are stained intensely with this antibody, allowing for easy quantification of proliferative and mitotic cells and identification of abnormal mitotic figures. Proliferative Index (PI) was calculated as the percentage of Ki67 positive cells out of the total number of epithelial cells. A minimum of 200 cells was counted in defined fields of view using a 40X objective. Likewise, Mitotic Index (MI) was calculated as the percentage of mitotic cells in the same fields of view. When no mitotic cells were observed, the MI was calculated as less than 1 mitotic cell per the total number of cells observed. Since the frequency of abnormal mitotic figures is very low in most tissues, the Abnormal Mitotic Index (AMI) was determined by scanning the entire section, counting the total number of mitotic cells and the total number of abnormal mitotic figures. The ratio of abnormal to total mitoses was then multiplied by the mitotic index to yield the AMI. These data are summarized in Table 1. All tissues were scored blindly. Photographs were made using a Nikon FXA photomicroscope.

## Centrin Immunofluorescence

A subset of tissues was selected for immunofluorescence studies. These tissues included one tumor with normal centrosome ultrastructure, one tumor with clusters of extra centrioles, two tumors with extra pericentriolar material, and two tumors with inverted polarity. Normal tissue used for immunofluorescence was from a different patient than that used in the ultrastructure studies. All tissues were frozen in liquid nitrogen within 30 minutes of surgical removal and stored at -70° until use. Cryosections were immunostained with a monoclonal antibody against centrin, a centrosomal protein, as previously described.[14] Sections were examined and

photographed using a Nikon FXA epifluorescence microscope.

## Results

### Normal Breast Epithelium

Normal breast epithelial tissues were organized with a high cuboidal layer of luminal cells separated at intervals from the basement membrane by a discontinuous layer of myoepithelial cells (Figure 1, A and B). The nuclei of the luminal epithelial cells tended to be basal and the centrioles apical. While apical, most often the position of the centrioles was eccentric; that is, they were located near the lateral junctional complexes of adjacent cells (Figure 1B). Although centrioles usually did not maintain an orthogonal orientation, they were typically close to each other (Figure 1, A and C). Occasionally, an extremely short primary cilium extended from the distal end of the mature centriole (Figure 1C). Fine striated rootlets infrequently were observed extending from the proximal ends of centrioles toward the base of the cell (Figure 1D). The striated rootlets were quite variable in extent and were not observed with most centrioles. Other than distal and subdistal appendages on the mature centriole and fine fibrillar material along the outer walls of the centriole barrels, little pericentriolar material was noted with the centrioles of normal luminal epithelial cells (Figure 1, A-D). Subdistal appendages were slightly more developed on the centrioles of the myoepithelial cells, and their primary cilia were longer than those of luminal epithelial cells (compare Figure 1, B and E). Unlike luminal epithelial cells, diplosomes of myoepithelial cells were located close to the nuclei. Filaments extended from the myoepithelial diplosome to the nucleus (Figure 1E); this was never observed in luminal epithelial cells. No centrosome abnormalities were observed in normal epithelial cells.

## Invasive Breast Tumors

Twenty-four of thirty-one invasive tumors contained centrosomes and that differed from those of normal breast cells in a variety of ways. Eleven tumors were characterized by centrosomes with more than two centrioles (Figures 2 and 3, A-C). In thin sections, these supernumerary centrioles ranged from a pair of centrioles with a single extra procentriole to a field of 9 centriole profiles (Figure 2, A-F). Often the extra centrioles were arranged in a group and were closely linked by fine fibers extending between subdistal appendages (Figure 2, C, E, and F). Appendages normally associated with only the mature centriole were seen frequently with more than one centriole in these groups (Figures 2, C-F and 3A). Centrosomes with extra centrioles were most often located adjacent to the nucleus (Figure 2, B, E, and F), in contrast to normal luminal epithelial cells in which the centrioles tended to be closer to the apical plasma membrane (Figure 1, A and B).

The amount of pericentriolar material and satellites associated with tumor centrosomes was variable, ranging from low levels similar to normal centrosomes (Figure 2, B-F), to moderate (Figure 2A) and excessive levels (Figure 3). In all, nine tumors had excess pericentriolar material, often in addition to extra centrioles. In some tumors this pericentriolar material had a distinct fibrogranular appearance (Figures 2A and 3) reminiscent of material associated with basal body formation in ciliated cells. Large granular masses, similar to generative complexes involved in ciliary basal body formation, were also observed in the pericentriolar material in some tumor cells (Figure 3E). Many centrioles were encased in electron opaque material appressed directly to the barrel of the centriole (Figure 3, B and C).

In addition to excessive pericentriolar material, two tumors had centrioles that were structurally defective in various aspects (Figure 4). Normal centrioles are composed of nine sets

of triplet microtubules in which the A microtubule is complete and the B and C microtubules share protofilaments with A and B respectively.[26] Unusual microtubule complexes were observed near complete centrioles in some tumors (Figure 4A). These microtubule complexes were not assembled into normal triplets nor arranged in a barrel shape; rather they were an assortment that included five or more microtubules with shared protofilaments embedded in amorphous electron opaque material (Figure 4A). In one instance a centriolar microtubule triplet was displaced away from the centriole barrel, resulting in what has been termed an "open ring" centriole (Figure 4B). Unusually long centrioles (Figure 4D) were observed in one tumor. Primary cilia ranged from very short to well developed (Figure 4C).

Some tumors had regions of apocrine metaplasia in which luminal epithelial cells maintained normal apical/basal polarity, but had cytoplasmic "beaks" that projected into the lumen (Figure 5A). The beaks were bordered by the apical plasma membrane that protruded well past the junctional complexes that mark the apical limit of the lateral plasma membrane. Beak cytoplasm contained numerous secretory vesicles, endoplasmic reticulum, and mitochondria. The centrosomes in these cells were near the junctional complexes and just apical to the nucleus, but not adjacent to the lumen as in normal luminal epithelial cells (Figure 5A). In one well-differentiated grade 2 tumor with apocrine metaplasia, the beaked apocrine cells were mixed with ciliated cells. The ciliated cells also maintained apical/basal polarity, but along their apical membrane were numerous cilia with centrioles functioning as ciliary basal bodies (Figure 5B). These cilia and basal bodies were similar in location and appearance to those of normally ciliated cells such as ciliated respiratory epithelium. Microvilli also were located along the apical membranes of the ciliated cells (Figure 5B). The apical membranes of the ciliated cells did not protrude into the lumen as did the non-ciliated beaked cells (Figure 5A). Both the

ciliated and the beaked cells were in regions of tumors that were well differentiated.

Two tumors contained regions in which cells still maintained apical/basal polarity even in poorly differentiated and highly invasive tumors lacking a basement membrane (Figure 5C). The apical and lateral membranes were identified by their location relative to junctional complexes and the presence of microvilli on the apical membrane. In these instances, the cell apices often did not face a lumen, but instead faced collagen fibrils of the stromal connective tissue (Figure 5C). The centrosomes of these cells were normal in structure and were located next to the junctional complexes near the apical plasma membrane, but since the apices face the stroma, the cell polarity is inverted.

## Mitosis in Tumor Cells

Although mitotic figures were not observed in normal breast tissues, there were numerous mitotic figures present in four of the tumors examined transmission electron microscopy. Some mitotic figures appeared normal in thin section, having a typical metaphase plate and bipolar spindle (not shown), while others had significant abnormalities (Figure 6). A tripolar mitosis is shown in Figure 6A. Tracings of microtubules, spindle poles, and condensed chromosomes from six non-adjacent serial sections through the cell in Figure 6A are presented in Figure 6B. Analysis of the reconstruction in three dimensions revealed that one spindle pole was composed of two distinct but adjacent foci of microtubules, which perhaps resulted from their coalescence in prometaphase. Many division figures were too bizarre for analysis in thin section.

## Proliferation and Mitotic Indices

Indices of proliferation (PI), mitosis (MI), and abnormal mitosis (AM) are summarized in Table

1. The one normal breast tissue examined by light microscopy had a mitotic index of less than 0.22%; i.e., no mitotic cells were present out of 456 cells observed, even though 19% of the cells in the same paraffin section were labeled with antibodies to Ki67. Nor were any abnormal mitotic figures observed upon examination of the entire section (~2x1 cm). Of the seven tumors with normal centrosome ultrastructure, two contained no abnormal mitotic figures while five did. Given the low mitotic index (<0.33% on average), the frequency of abnormal mitoses in tumors with normal centrosome ultrastructure is less than 0.08%. The PI of the tumors with normal centrosomes was not significantly different from that of normal tissues (18 and 19%, respectively).

The 21 tumors with abnormal centrosomes had a mean PI of 26%. One tumor, characterized by the presence of open ring centrioles, had a PI of only 1% and no mitotic figures were present in the section examined (~1x1 cm). The remaining 20 tumors with abnormal centrosome ultrastructure had both normal and abnormal mitotic figures present (Figures 6 C and D). The mean MI for the 21 tumors was 0.63%, with a range of 0.19 – 1.77%, and the mean AMI was 0.29%, with a range of 0 – 0.87%. Tumors with extra pericentriolar material tended to have higher AMIs than did other tumors (0.44% as opposed to 0.29%), although the ranges had a large overlap.

## Centrin Immunofluorescence

As previously described[14], normal breast tissues have an apically positioned pair of immunolabeled spots that correspond to the centrioles (Figure 6E). Pairs of spots also were observed in cells of the tumor with normal centrosome ultrastructure, although the tissue was anaplastic and centriole location appeared random (Figure 6F). Many cells in the tumor with

numerous centrioles closely linked by fine fibers contained clusters of spots the size and shape of centrioles (Figure 6G), whereas spots of various sizes and shapes were present in cells of the tumors characterized by extra pericentriolar material (Figure 6H).

## Discussion

The centrosome functions to nucleate and organize microtubules; during interphase the centrosome is the primary microtubule organizing center and during mitosis duplicated centrosomes serve as mitotic spindle poles.[19] We found that centrosomes in normal breast tissue are apical and usually adjacent to the junctional complex, while nuclei are basal. Very little pericentriolar material is associated these centrosomes. As is seen in other polarized epithelial cells,[27] centrioles may separate a short distance from each other after losing their orthogonal orientation, and the mature centriole may form a short primary cilium. In addition, centrioles occasionally bear a striated rootlet.

Only by selecting breast biopsy tissue from pre-menopausal women in the luteal phase of the menstrual cycle was Ferguson[28] able to investigate mitosis in normal breast parenchyma. In these normal cells, very little pericentriolar material was associated with the spindle poles. The normal tissues in the present study were not selected according to the phase of menstrual cycle, and no mitoses were observed by transmission electron microscopy or by light microscopy. However, normal breast epithelium does maintain a population of proliferating cells that immunostain with antibodies to Ki67; our PI value of 19% in normal breast epithelium is within the range of published values[29]. In agreement with our observations on interphase cells by immunofluorescence and by transmission electron microscopy, Ferguson[28] noted that centrioles of normal interphase cells were apical and not associated with the basal nuclei. Likewise,

primary cilia have previously been noted in myoepithelial cells.[30]

Centrosomes undergo changes throughout the cell cycle.[21-24] The nuclear and centrosome cycles are synchronized by checkpoints that prevent DNA reduplication prior to karyokinesis and prevent centrosome reduplication prior to anaphase. In certain normal cell types such as binuclear mouse hepatocytes[31] and human megakaryocytes,[32] synchrony between the nuclear and centrosome cycles is maintained even in the absence of cytokinesis, resulting in polyploid cells with centrosome numbers appropriate for the level of ploidy. Due to the numerous centrosomes arranged around the polyploid nucleus, megakaryocytes lack apical/basal polarity, although they do have a radial organization. In contrast, cancer cells have asynchronous nuclear and centrosome cycles, often resulting in multi-centrosomal aneuploid cells that lack apical/basal polarity and appear disorganized.

We have shown that centrosomes and centrioles of most human breast tumors (24 of 31 analyzed) display a range of significant structural and functional abnormalities, and some of these abnormalities are associated with increased frequencies of abnormal mitoses. In general, only one kind of structural abnormality is present in a given tumor. Cells having no visible centrosome abnormality are also present in tumors. Some abnormalities may be related to loss of synchrony between the centrosome cycle and nuclear cycle.

Tumor cells that become ciliated retained apical/basal polarity and tended to be well differentiated. Ciliated cells have been described infrequently in breast carcinomas.[33] These multiple centrioles probably arise through the same acentriolar basal body neogenesis that occurs in normal ciliated epithelial cells.[34-37] In effect, these cells differentiate into the wrong cell type, resulting in metaplasia rather than anaplasia. These ciliated breast tumors have proliferative and mitotic indices (20% and 0.2%, respectively) similar to normal breast epithelium. The ciliated

cells, like normal ciliated epithelial cells, probably are terminally differentiated and remain in G0 of the cell cycle. Therefore, the production of centrioles that function as ciliary basal bodies may be a relatively harmless structural alteration with no adverse implications for genetic stability.

"Open-ring" centrioles and centrioles missing triplet MTs occur in some tumors. Although these structures are similar to those present during basal body formation in hamster ciliogenesis,[38] no cilia are present in these tumors. Disrupted centriole barrels similar to open-ring centrioles have also been observed as a consequence of infection with and treatment with DNA binding dyes[39], and DNA binding dyes have been shown to induce multipolar mitoses in cultured cells.[39] However, in the present study, open ring centrioles are not associated with an increase in the frequency of multipolar mitoses.

Unusual microtubule complexes embedded in dark amorphous material were also noted in one tumor. The proliferative, mitotic, and abnormal mitotic indices of this tumor are not significantly different from that of tumors with normal centrosome structure. These novel structures have not been described previously, and their importance is not understood. They may be a further indication that the mechanics, as well as timing, of centriole formation is not well regulated in tumors.

Some tumors (11 of 31 studied) produce extra centrioles that do not serve as ciliary basal bodies. In these cells, the centrioles often appear linked closely together by fine fibers and remain near the nucleus. These tumors are anaplastic; i.e. they are not as differentiated as tumors that produce cilia and do not retain apical/basal cell polarity. The presence of procentrioles along the proximal walls of mature centrioles indicates that these extra centrioles arose through template driven duplication rather than through acentriolar neogenesis typical of basal body production in ciliated cells.[35] Fine fibers linking the centrioles in tumors are similar to those

described linking the pair of centrioles of a diplosome,[40] further supporting the idea that they originate as procentrioles associated with a mature centriole. Since template driven centriole duplication normally occurs only once per nuclear cycle, these cells have lost the synchrony between the nuclear cycle and the centrosome cycle. As long as the centrioles remain linked together, they may function as one large centrosome in an interphase cell. However, if these large centrosomes separate into more than two spindle poles at the onset of mitosis, it is likely that chromosomal mis-segregation will occur, resulting in aneuploidy. Indeed, the frequency of abnormal mitoses is quite variable among these tumors, indicating that most cells with extra centrioles are capable of forming bipolar spindles.

Other tumors (9 of 31 studied) accumulate excess pericentriolar material with their centrosomes and variable numbers of extra centrioles. The nature of the pericentriolar material is reminiscent of fibrogranular material and generative complexes associated with acentriolar as well as centriolar basal body formation.[34-37, 41] However, no cilia are observed and the randomly positioned centrioles are not located near the plasma membrane. This accumulation of excess pericentriolar material may be the result of overexpression of centrosomal proteins or the reorganization of material that is normally dispersed within the cytoplasm.[14; 42; 43] Increased levels of γ-tubulin[14; 17], pericentrin[15], and centrin[14] have been demonstrated in abnormal centrosomes in human tumors, and it is likely that other centrosomal proteins are present in increased levels as well. Gamma-tubulin containing complexes located in the pericentriolar material are the site of microtubule nucleation, and as such are key to centrosome function.[44] We have shown that tumors with excess pericentriolar material are highly anaplastic and have lost cell polarity. These tumors tend to have higher AMIs (0.44% on average) compared to tumors with other centrosome abnormalities (0.22% on average). A higher frequency of abnormal

mitoses in tumors with extra pericentriolar material suggests that the regulation of accumulation of centrosomal proteins is more critical than regulation of centriole duplication for proper centrosome function during the cell cycle.

Some cells have more than two centrosomes that can function as spindle poles, yielding atypical multipolar mitoses. Atypical mitoses have been observed in breast tumors and other pathological specimens such as ulcerative colitis[7] and a mouse model of pancreatic cancer.[3] Multipolar mitoses were observed in several breast tumors in the present study. Aberrant mitoses such as these may arrest in metaphase, with the cells eventually undergoing apoptosis. In some instances, however, a selective advantage may be conferred to one of the daughter cells, leading to a clone of cells with chromosome gains and/or losses.

Serial sectioning through mitotic tumor cells showed that spindle poles are sometimes composed of more than one focus of microtubules. These spindle poles likely resulted from the coalescence of two or more centrosomes prior to metaphase. Coalescence of centrosomes could allow the formation of a bipolar spindle in a cell having extra centrosomes. Coalescence of extra centrosomes may be a mechanism by which cells can minimize the rate at which aneuploidy develops in tumors. Since compounded aneuploidy ultimately would be a self-limiting characteristic of tumors, a proportion of bipolar mitoses must be maintained for tumor growth.

The centrosomal abnormalities described here in breast tumor cells reflect changes in the status of cell and tissue differentiation of the tumors. Differentiated tumors have centrosomes of more normal appearance that are either mis-located, as in the tumors with inverted cell polarity, or perform a normal function not typical of mammary epithelial cells, such as producing ciliary basal bodies in tumors displaying apocrine metaplasia. Centrosome abnormalities are characteristic of poorly differentiated anaplastic tumors that have lost checkpoint

synchronization of nuclear and centrosome cycles. This loss is reflected in centrosome defects and multipolar mitoses. As recognized by Boveri[2] earlier in this century, defective centrosomes may decrease the fidelity of chromosome segregation during multipolar mitoses. Consequently, centrosome abnormalities such as those described here may confer a "mutator phenotype" to tumor cells. As is the case for the molecular mutator phenotype, most mutated progeny will not be viable, but occasionally progeny with a selective advantage will emerge and thrive, and thus the tumor progresses to a more aggressive state.

## Acknowledgments

The authors thank Ms. Denise Morgan and Ms. Belinda Hoebing of the Surgical Pathology Laboratory and the staff of the Electron Microscopy Core Facility for collecting and processing tissues used in this study and Ms. Linda Murphy for preparation of the Ki-67 immunostained slides.

## Table I

| tissue | centrosome structure | PI[a] | range | max MI[b] | range | AMI[c] | range | N |
|---|---|---|---|---|---|---|---|---|
| normal | normal | 19% | NA | 0.22% | NA | 0.00% | NA | 1 |
| tumor | normal or abnormal | 24% | 1 - 77% | 0.55% | 0.19 - 1.77% | 0.24% | 0.00 - 0.87% | 28 |
| tumor | normal | 18% | 5 -29% | 0.33% | 0.25 - 0.35% | 0.08% | 0.00 - 0.19% | 7 |
| tumor | all abnormal | 26% | 1 - 77% | 0.63% | 0.19 - 1.77% | 0.29% | 0.00 - 0.87% | 21 |
| tumor | abnormal, no extra pcm[d] | 20% | 1 - 54% | 0.51% | 0.19 - 1.77% | 0.22% | 0.00 - 0.87% | 13 |
| tumor | extra pcm | 34% | 18 - 77% | 0.83% | 0.27 - 1.55% | 0.44% | 0.08 - 0.85% | 8 |

[a] Proliferative Index calculated as percent of epithelial cells labeled with Ki-67 antibodies.

[b] Mitotic Index calculated as percent of mitotic epithelial cells. For tissues with no mitotic cells in the fields counted, the percent is calculated as <1 in the total number observed.

[c] Abnormal Mitotic Index calculated as percent of epithelial cells with abnormal mitotic figures.

[d] Pericentriolar material

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## Figure Legends

**Figure 1. Normal Breast Epithelium. A**. The normal breast ductal epithelium consists of a high cuboidal layer of luminal cells subtended by a discontinuous layer of myoepithelial cells (*) and basement membrane (arrows). The nuclei (N) are basal and the centrosomes (circled) are apical. **B**. Adjacent luminal epithelial cells are joined by lateral junctional complexes (brackets) near the apical membrane and desmosomes (arrows) between their lateral membranes. A single centriole (arrowhead) is located at the apex next to a junctional complex. A portion of a myoepithelial cell (M) is seen at the base of the luminal epithelial cell. **C**. The mature centriole of this non-orthogonal diplosome bears a short primary cilium (arrow) at its distal end in this luminal epithelial cell. A small subdistal appendage (arrowhead) is present on the mature centriole, while the immature centriole lacks appendages and a primary cilium. Very little pericentriolar material is present. **D**. A striated rootlet extends from the proximal end of this mature centriole toward the base of the luminal epithelial cell. E. Fine fibers (small arrowhead) extend between the diplosome and the nearby nucleus (N) in this myoepithelial cell. Distal appendages (large arrowhead) extend between the centriole and the plasma membrane at the site of primary cilium (large arrow) emergence. Subdistal appendages (small arrow) are prominent on the mature centriole. The immature centriole is seen in oblique section. Magnifications: **A** X 3500; **B** X 8850; **C** X 27,500; **D** X 25,600; **E** X 21,200.

**Figure 2. Supernumerary Centrioles in Breast Tumors. A**. A procentriole (arrow) is present at the proximal end of one of the two centrioles in this section. This procentriole is identifiable by its orthogonal orientation relative to the full length centriole and by the width of its lumen. Notice the electron opaque pericentriolar satellites surrounding the centrioles. **B**. Two centrioles

are seen in cross section and a third is in longitudinal section. One centriole has subdistal appendages (arrow). All three are close to the nucleus (N). There is no orthogonal relationship between any of the three centrioles. **C**. At least two of these four centrioles have subdistal appendages (arrows). **D**. The barrels of these five centrioles are coated with a fine electron opaque material. Two centrioles have distal appendages (arrows) and at least one also has subdistal appendages (arrowheads). **E**. This group of six centrioles is linked by fine fibers between their subdistal appendages (arrows). The group is next to the nucleus (N). **F**. At least nine centriole profiles are present in this thin section. Subdistal (arrows) and distal (arrowheads) appendages are seen on many of the centrioles. The nucleus (N) is adjacent to this cluster of centrioles. Magnifications: **A** and **B** X 27,500; **C** and **F** X 32,300; **D** X 31,000; **E** X 34,150.

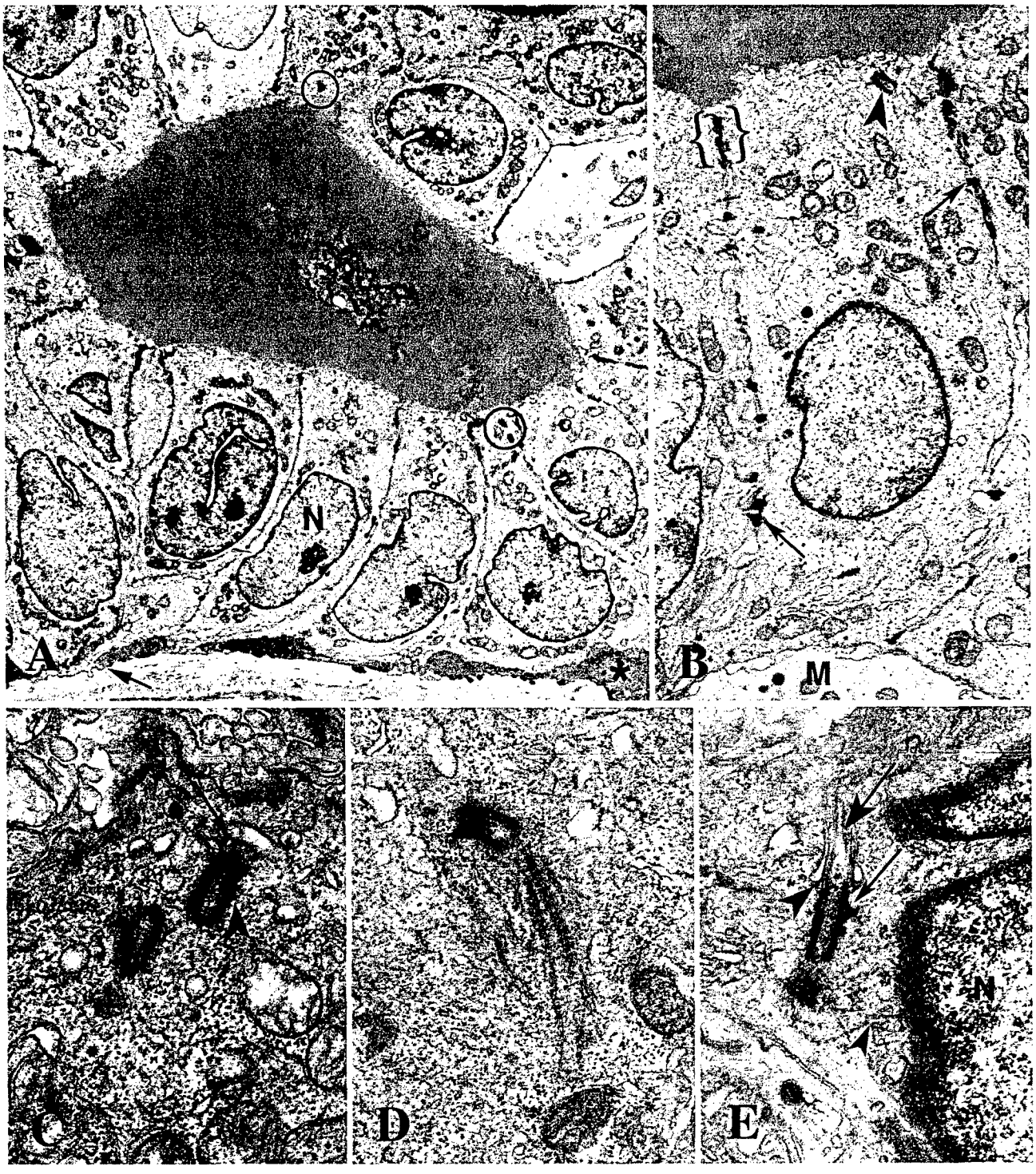
**Figure 3. Excess Pericentriolar Material in Breast Tumors. A**. Centrosomes in two adjacent cells are seen. Desmosomes (small arrows) tether the plasma membranes. All of the centriole profiles include subdistal appendages that are characteristic of mature centrioles (large arrows). Electron opaque fibrogranular material is present around both centrosomes. **B**. The barrels of these centrioles are coated with a dark granular material and pericentriolar satellites are present. One centriole has distal and subdistal appendages (arrows) while the other has a procentriole (arrowhead) associated with it. **C**. Fine electron opaque fibers coat the five centriole profiles seen in this section. Two orthogonal centrioles are connected by a dense parallel array of fibers (arrow). **D**. Two centrioles with numerous dark granules and fibrous material are present in this section. **E**. This centrosome contains one centriole and several masses (arrows) similar to generative complexes visible in this section. Magnifications: **A** X 17,900; **B** X 31,650; **C** X 28,000; **D** X 28,700; **E** X 27,650.

**Figure 4. Abnormal Centriole Structure in Breast Tumors.** **A**. Subdistal appendages are seen in this oblique section through a centriole. Numerous microtubule complexes (large arrows) are seen in various planes of section throughout the cytoplasm near the centriole. As is seen in cross section of the complexes, the individual microtubules share a portion of the wall of the neighbor microtubules (small arrow). **B**. The open-ring configuration of this centriole is shown in cross section. Two of the nine triplet microtubule complexes are splayed away from the centriole barrel (arrow). **C**. This centriole bearing a primary cilium (*) is nearly twice as long as normal centrioles. Magnifications: **A** X 54,500; **B** X 59,625; **C** X 47,700.

**Figure 5. Positional Centrosomal Anomalies in Breast Tumors.** **A**. Secretory granules (arrows) are present at the apical membrane of these cells displaying apocrine metaplasia. Junctional complexes (brackets) mark the transition from lateral to apical membrane domains. Apocrine beaks extend into the lumen of the duct. Notice the centriole (circled) near the apical end of the nucleus. These cells have apical/basal polarity and rest on a basement membrane (arrowheads). **B**. Extra centrioles in this cell are inserted at the apical plasma membrane where they function as basal bodies (arrows) for cilia (small arrows). Microvilli and cilia project into the lumen. The beak of an adjacent apocrine cell (*) is visible. The ciliated cell does not protrude into the lumen, as does the apocrine cell; but like its apocrine neighbor, it has apical/basal polarity and rests on a basement membrane (not visible in this figure). **C**. The two centrosomes (arrows) seen in adjacent cells are located near the junctional complex between these polarized cells (bracket). However the apical membrane domain with microvilli faces collagen (*) of the stromal tissue rather than the lumen of a duct. This invasive group of cells has ramified through the breast stroma and is not subtended by a basement membrane. The polarity of these cells is

inverted, with the basal domains abutting the basal domains of other cells and the apical domains facing the stroma rather than a lumen. Magnifications: **A** X 8150; **B** X 10,000; **C** X 7900.

**Figure 6. Multipolar Mitoses and Centrin Immunofluorescence.** **A**. This section through a symmetrical tripolar mitotic cell shows part of the metaphase plate and portions of the tripolar spindle. **B**. Tracings of microtubules, spindle poles, and condensed chromosomes from six non-adjacent serial sections through the cell shown in Figure 6A are shown in this overlay. At least two centrioles are present at each spindle pole. The upper spindle pole appears to contain two separate, but adjacent, microtubule foci that have coalesced. **C**. A normal metaphase plate is shown in this Ki-67 immunostained paraffin section of a breast tumor. **D**. A tripolar metaphase cell immunolabeled with Ki-67 is shown in this tumor section. **E**. In normal breast epithelium, the centrosomes appear as distinct pairs of spots when labeled with antibodies against centrin. Centrosomes of two adjacent cells are shown in this cryosection. **F**. In this tumor characterized with normal centrosome ultrastructure, the centrosomes are similar to those of normal tissue when immunolabeled using antibodies against centrin. **G**. Centrin immunofluorescence of the same tumor shown in Figures 2 E and F reveals a cluster of centriole-sized spots as well as a normal looking pair of spots. By transmission electron microscopy this tumor had up to 9 centrioles in a single thin section, but no excess pericentriolar material. **H**. Centrin immunofluorescence of the same tumor as shown in Figures 3D and 6A reveals numerous large, amorphous spots. By transmission electron microscopy, centrosomes of this tumor contain excess pericentriolar material and extra centrioles. **A** and **B** X 2160; **C** and **D** X 925; **E – H** X 2050.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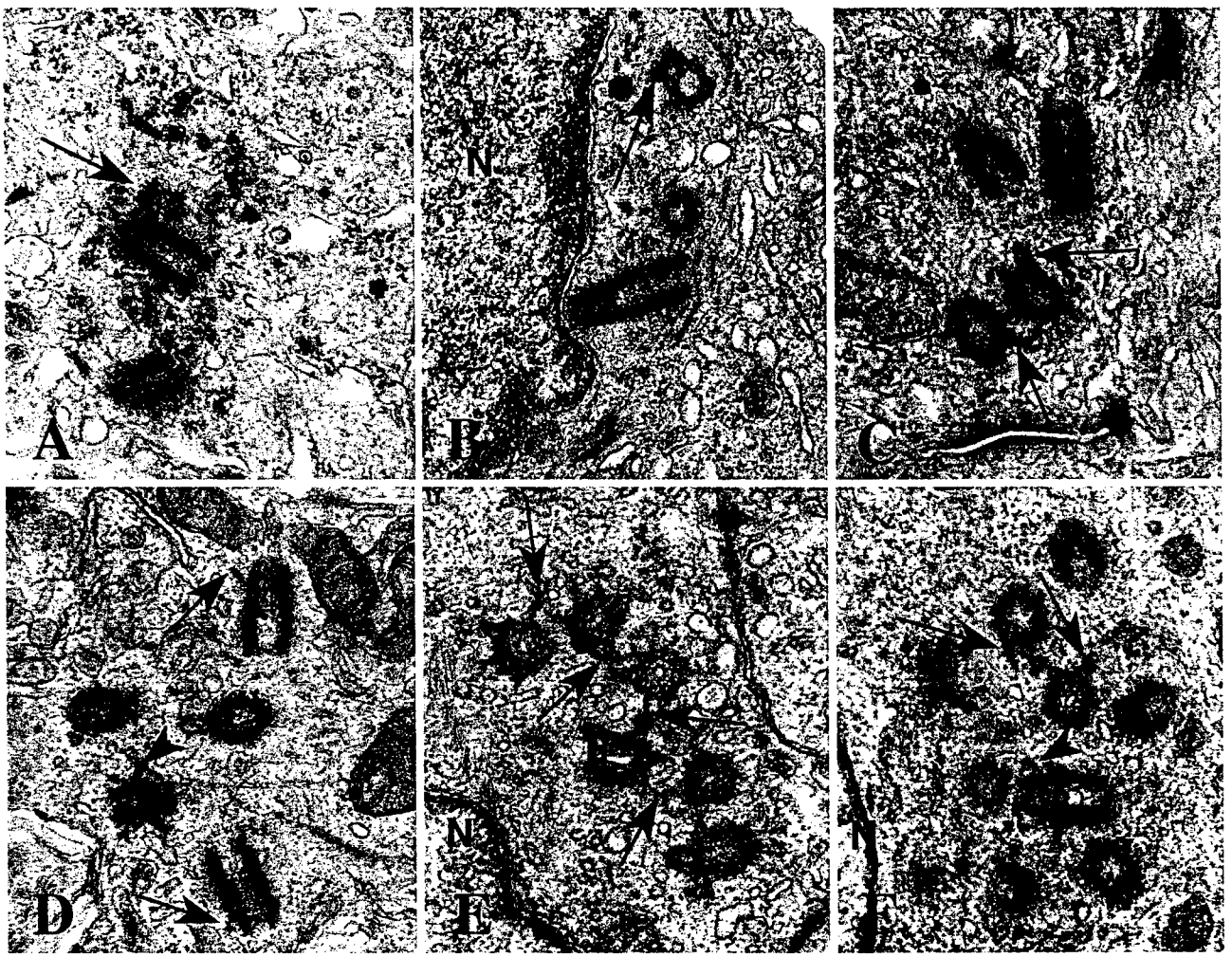
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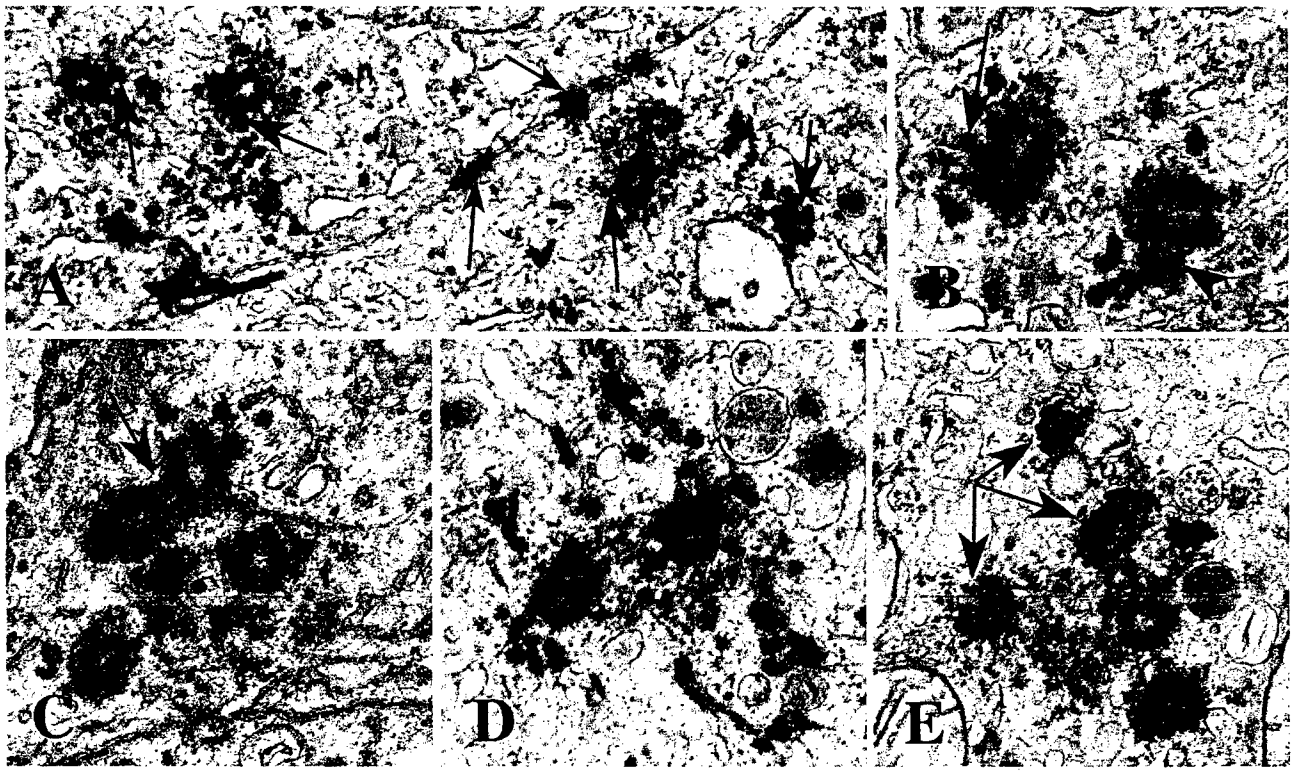
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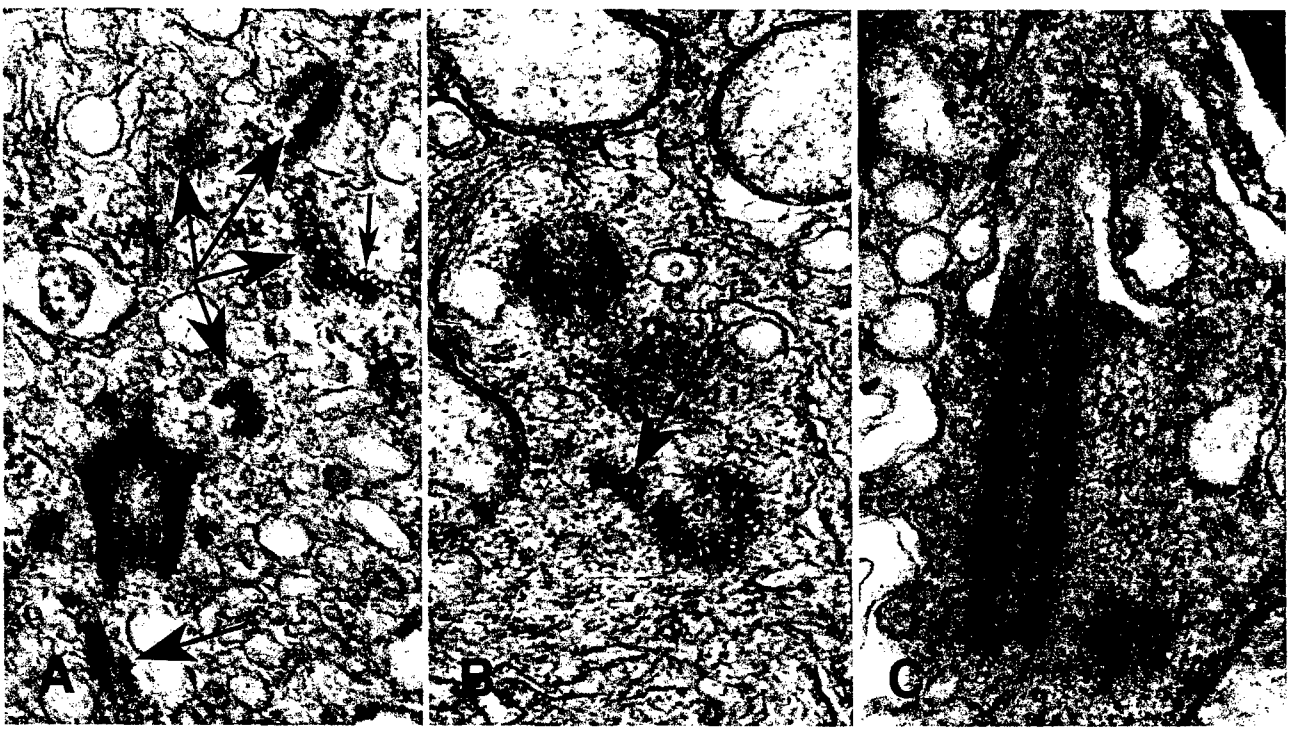
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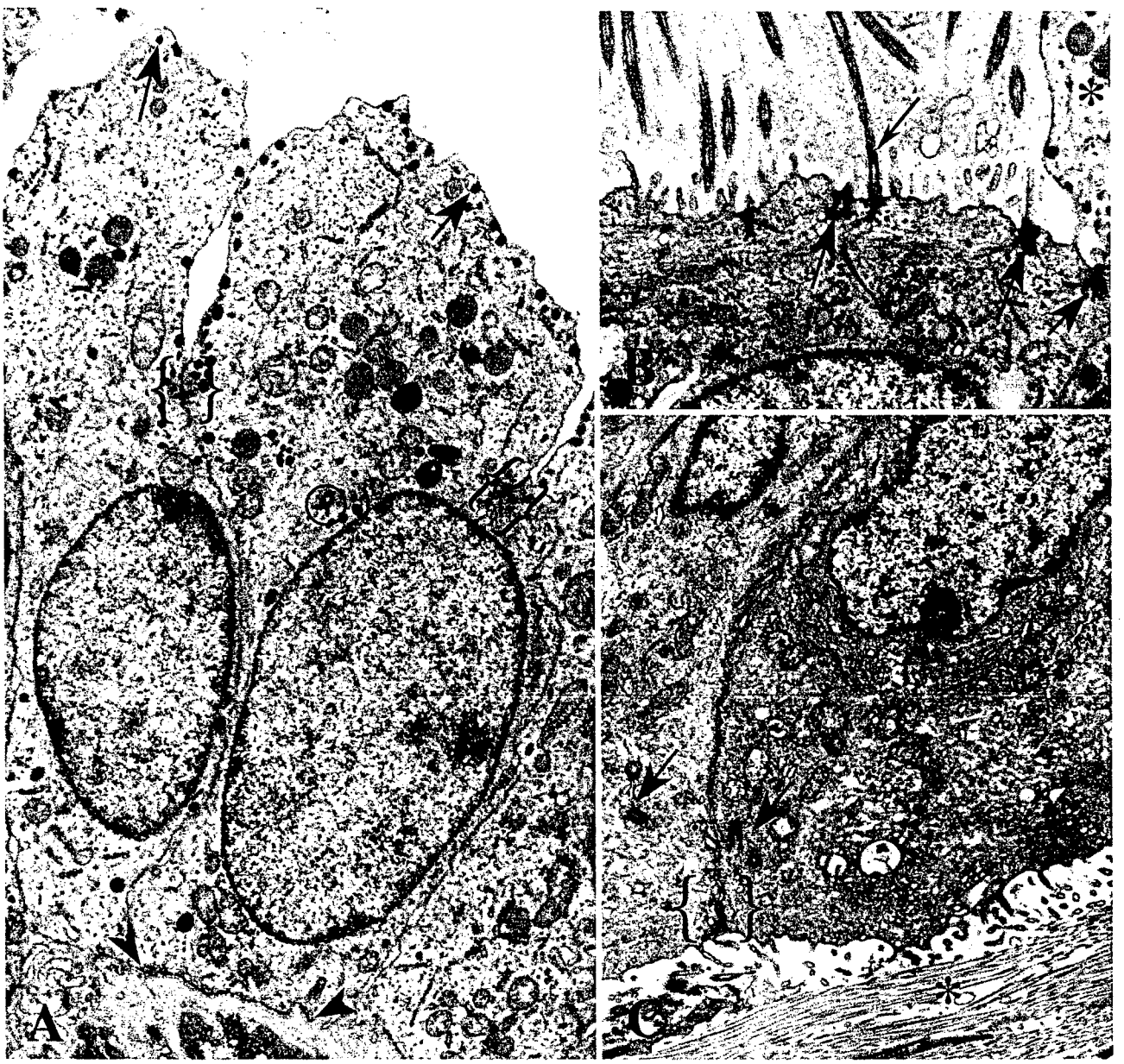
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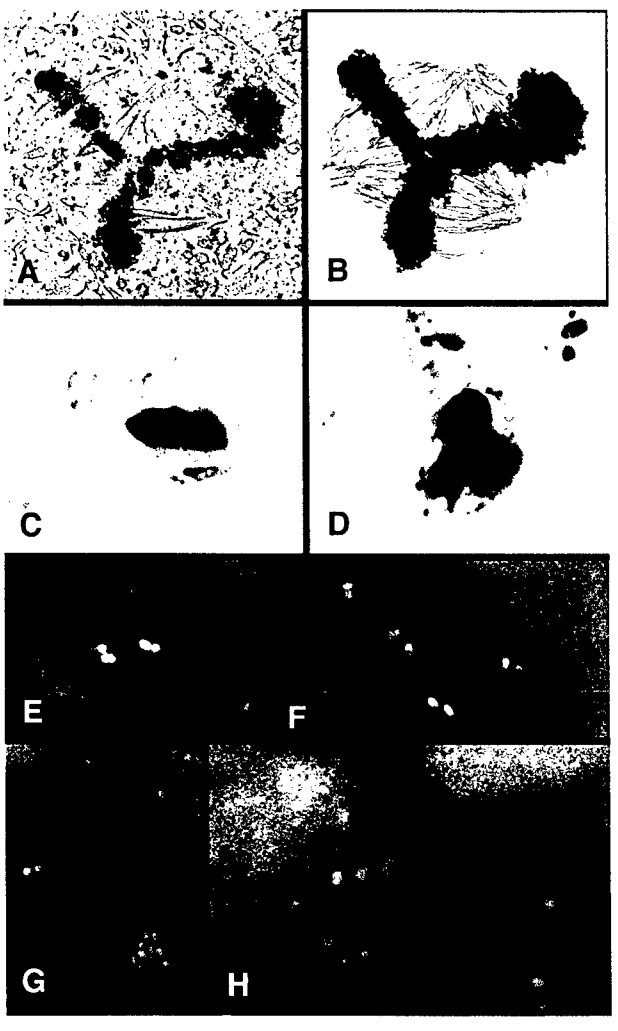
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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MD 21702-5012

REPLY TO
ATTENTION OF

MCMR-RMI-S (70-1y) 15 May 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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